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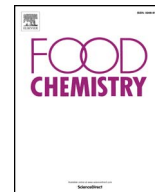
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Discrimination of processing grades of olive oil and other vegetable oils by monochloropropanediol esters and glycidyl esters

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2-Chloropropane-1,3-diol (PubChem CID: 10337)
 3-Chloropropane-1,2-diol (PubChem CID: 7290)
 Glycidol (PubChem CID: 11164)
 Diacylglycerol (PubChem CID: 60,26,790)
 Methanol (PubChem CID: 887)
 1234-Tetrachloronaphthalene (PubChem CID: 29910)
 Iso-octane (PubChem CID: 10907)
 Tetrahydrofuran (PubChem CID: 8028)
 n-Heptane (PubChem CID: 8900)
 Acetone (PubChem CID: 180)
 Toluene (PubChem CID: 1140)
 Sulfuric acid (PubChem CID: 1118)
 Sodium hydrogen carbonate (PubChem CID: 516892)
 Sodium sulphate (PubChem CID: 24,436)
 Sodium bromide (PubChem CID: 2,53,881)
 Phenylboronic acid (PubChem CID: 66,827)

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ABSTRACT

In this study, the processing derived contaminants 2- and 3-monochloropropanediol (2- and 3-MCPD) esters and glycidyl esters (GEs) were analysed in 84 oil samples by GC–MS/MS for the discrimination of processing grades of olive oils as a potential authentication tool. Concentrations of 2- and 3-MCPD esters and GEs varied in the ranges 0–6 mg/kg, 0–1.5 mg/kg, and 0–1 mg/kg oil, respectively. The concentrations of the three compounds in lower grade olive oils were significantly higher ($P < .001$) than that in EVOO. A similar difference was observed for other refined and cold-pressed vegetable oils. The limit of fraud detection of lower grade oils in EVOO was 2% when using 3-MCPD esters, 5% for 2-MCPD esters, and 13–14% for GEs based on calculations of virtual mixtures of the current sample set. Especially the MCPD esters appear very specific and promising for the detection of lower processing grade oils in EVOO.

1. Introduction

In recent decades, the popularity of olive oil has seen a rise worldwide due to its perceived health benefits. This holds especially for extra virgin olive oil (EVOO). The easiness to adulterate EVOO, the difficulty of detection, discrepancy between supply and demand, economic drivers, as well as cultural and behavioural risk factors and lack of control measures contribute to the susceptibility of EVOO to fraud. Three typical olive oil frauds have been reported: (a) the most common

way is to blend with other vegetable oils (Jabeur et al., 2014), (b) replacement with vegetable oil with addition of chemical compounds to disguise the adulteration (Roca, Gallardo-Guerrero, Minguez-Mosquera, & Rojas, 2010), and (c) the replacement of EVOO with lower olive oil grades. The latter may include refined olive oil (ROO) (Karbasian, Givianrad, & Ramezan, 2015) and pomace olive oil (POO) (Škevin et al., 2011), as well as soft deodorised oils (Aparicio-Ruiz, Romero, García-González, Oliver-Pozo, & Aparicio, 2017).

Since EVOO adulteration is a serious issue, it is desirable to explore

Abbreviations: 2-MCPD, 2-chloropropane-1,3-diol; 3-MCPD, 3-chloropropane-1,2-diol; C-VEGE, cold pressed vegetable oil; EVOO, extra virgin olive oil; GC–MS/MS, gas chromatography tandem mass spectrometry; GEs, glycidyl esters; JECFA, the Joint FAO/WHO Expert Committee on Food Additives; MCPD, monochloropropanediol; MeOH, methanol; PBA, phenylboronic acid; POO, pomace olive oil; ROO, refined olive oil; R-VEGE, refined vegetable oil; TDI, tolerable daily intake

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innovative and reliable methods to reveal EVOO fraud. In order to guarantee food quality and expose olive oil fraud issues, many analytical strategies have been reported over the last few decades (Luykx & Van Ruth, 2008). The chemical methods are widely used to identify olive oil adulteration and are based on single or multiple markers (Tres & van Ruth, 2011). Those markers can be divided into two groups. The first one comprises the major components of olive oils, such as triglycerides (Jabeur et al., 2014), fatty acids (Škevin et al., 2011), waxes and sterols (Aparicio & Aparicio-Ruiz, 2000). The second group consists of minor components of olive oils, and include chlorophylls (Roca et al., 2010), carotenoids (Moyano, Melendez-Martinez, Alba, & Heredia, 2008), phenolic compounds (Lerma-García, Herrero-Martinez, Ramis-Ramos, & Simo-Alfonso, 2008), and squalene (Ben Mansour et al., 2015).

The detection of ROO in EVOO remains a challenge though. Although one would usually look for the reduction of the desirable EVOO compounds, the naturally present variation of those compounds would still result in a relatively wide acceptable range. Since many compounds are removed during the refining process, very few unique characteristics are left. The only ones to be considered are compounds that are formed in the refining process and are persistent throughout all the steps in the refining process. Monochloropropanediol (MCPD) esters and glycidyl esters (GEs) may be that kind of compounds, but few studies have looked into these compounds in olive oils so far.

MCPD esters are minor compounds derived from diacylglycerols and are chlorinated through refining processes and special treatments. Free 3-MCPD was reported first in acid-hydrolysed vegetable proteins (Velisek et al., 1980). It was also detected at an early stage in rapeseed oil adulterated with aniline and refined with hydrochloric acid (Gardner et al., 1983). GEs are minor compounds formed from monoacylglycerols in the refining process. Although many studies looked into the formation of MCPD esters and GEs, a definite, and generally accepted mechanism has not been established yet (Zhao et al., 2016). Some studies have focused on factors that influence the formation of those compounds. According to Weißhaar (2008), temperature, pressure, water activity and other technical parameters during the refining process trigger the formation of 3-MCPD esters. Deodorization, a process which requires usually high temperatures, result in considerable formation of 3-MCPD esters and GEs especially when the temperature reaches up to 230 °C (Hrnčirik & van Duijn, 2011). Zelinkova, Svejkovska, Velisek, and Dolezal (2006) reported also an increase during prolonged heating of rapeseed oils at 230 °C, but in contrary a reduction under the same conditions for olive oils.

MCPD and glycidol are not harmless compounds. Various toxicological studies revealed their toxicity. The International Agency for Research on Cancer (IARC) characterizes 3-MCPD as possibly carcinogenic to humans, based on cancer incidents caused by 3-MCPD in laboratory animals (IARC, 2012). The European Food Safety Authority (EFSA, 2016) concluded that the critical effect of 3-MCPD is kidney toxicity and glycidol has potential genotoxic and carcinogenic effects. Meanwhile, the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2016) concluded that glycidol is a genotoxic compound. Based on those research data, the EFSA (2016) established a tolerable daily intake (TDI) of 0.8 µg/kg bw per day for the sum of free 3-MCPD and 3-MCPD esters. More recently the JECFA (2016) announced a regulatory maximum TDI of 4 µg/kg body weight (bw) per day for free 3-MCPD and 3-MCPD esters. In view of insufficient toxicokinetic data, no health-based guidance value could be established for 2-MCPD. Due to genotoxicity and carcinogenicity, it is not appropriate to establish a health-based guidance value for glycidol. Therefore, the margin of exposure (MoE) approach was applied, MoE estimates were calculated by dividing the reference point of T25 10.2 mg/kg bw per day by the exposure levels (EFSA, 2016; JECFA, 2016). MCPD and glycidol may be useful for discrimination of processing grades of oils, but we have to consider that there is also an unusual dark side to this group of markers.

In the current study, we aim to explore the processing derived

contaminants MCPD esters and GEs for discrimination of processing grades of olive oils as potential authentication tool. The results will be compared to the levels of these compounds in some other vegetable oils.

2. Materials and methods

2.1. Samples

Eighty-four oil samples, the authenticity of which was confirmed in various preliminary tests, which are based on fatty acid compositional fingerprinting, volatile organic compound fingerprinting, and spectroscopic tests measuring K232, K268 and ΔK values (IOC, 2015), were selected from a pool of 400 oil samples of a PhD project. The large oil set was supplied by many OO producers, traders, and retailers in the EU. For the current study, the selected set of 84 samples comprised 30 EVOO samples (Origin: 6 Greece, 14 Italy, 6 Spain, 4 EU), 18 ROO samples (Origin: 4 Italy, 5 Spain, 9 EU), 16 POO samples (Origin: 6 Italy, 6 Spain, 4 EU), 8 cold pressed vegetable oil samples (C-VEGE) (Origin: 8 EU), and 12 refined vegetable oil samples (R-VEGE) (Origin: 1 South America, 1 Africa, 10 EU). C-VEGE consisted of 7 cold-pressed rapeseed oils and 1 sunflower oil, and R-VEGE consisted of 3 refined rapeseed oils, 4 peanut oils, and 5 sunflower oils.

Blends of one EVOO and one ROO sample, as well as one EVOO and one POO sample were manually prepared (mix1 and mix2) and comprised 10%, 20%, 30%, 40% and 50% of ROO or POO in EVOO.

Prior to analysis, samples were stored in capped bottles, which were kept in the dark at room temperature until analysis.

2.2. Reagents and standards

2.2.1. Reagents

Tetrahydrofuran, anhydrous; methanol, analytical grade; n-heptane, analytical grade; acetone, analytical grade; toluene, analytical grade; sulfuric acid (purity ≥ 95%); sodium hydrogen carbonate (purity ≥ 99%); sodium sulphate (purity ≥ 99%); phenylboronic acid (purity ≥ 97%); sodium bromide (purity ≥ 99.5%), all reagents upon were purchased from Sigma-Aldrich. Ultra-pure water obtained from a Millipore Milli-Q purification system (Millipore Co., Bedford, MA, USA). 1,2,3,4-tetrachloronaphthalene (1,2,3,4-TCN) 10 µg/mL in iso-octane were obtained from Agilent.

2.2.2. Standards

1,2-Bis-palmitoyl-3-chloropropanediol [PP-3-MCPD] (98%, CAS: 51930-97-3), 1,3-dipalmitoyl-2-chloropropanediol [PP-2-MCPD] (98%, CAS: 169471-41-4), and glycidyl palmitate [Gly-P] (98%, CAS: 7501-44-2) were all purchased from Toronto Research Chemicals (Toronto, Canada). The stock solutions of 1 mg/mL in toluene were prepared for those three native standards PP-3-MCPD, PP-2-MCPD, and Gly-P.

1,2-Dipalmitoyl-3-chloropropanediol-d5 [PP-3-MCPD-d5] (1.0 mg/mL in Toluene) (99.5%, CAS: 1185057-55-9), 1,3-dipalmitoyl-2-chloropropanediol-d5 [PP-2-MCPD-d5] (1.0 mg/mL in Toluene) (97.4%, CAS: 1426395-62-1), and glycidyl palmitate-d5 [Gly-P-d5] (1.0 mg/mL in Toluene) (99.5%, CAS: 1794941-80-2) were purchased from Chiron AS (Trondheim, Norway), supplied by Campro Scientific (Veenendaal, The Netherlands).

For preparation of the working solutions for the calibration curve, a modified method of the AOCS Cd29a_13 method (AOCS, 2013) was followed, the changes are listed in Table 1S.

2.3. Sample preparation

The general principle of this method is the conversion of glycidyl esters into 3-MBPD esters with sodium bromide and diluted sulphuric acid up front, which is then followed by an acid catalysed hydrolysis of all esters (16 h, 40 °C, with sulphuric acid in methanol) in order to release the bound contaminants (2-MCPD, 3-MCPD, and glycidol).

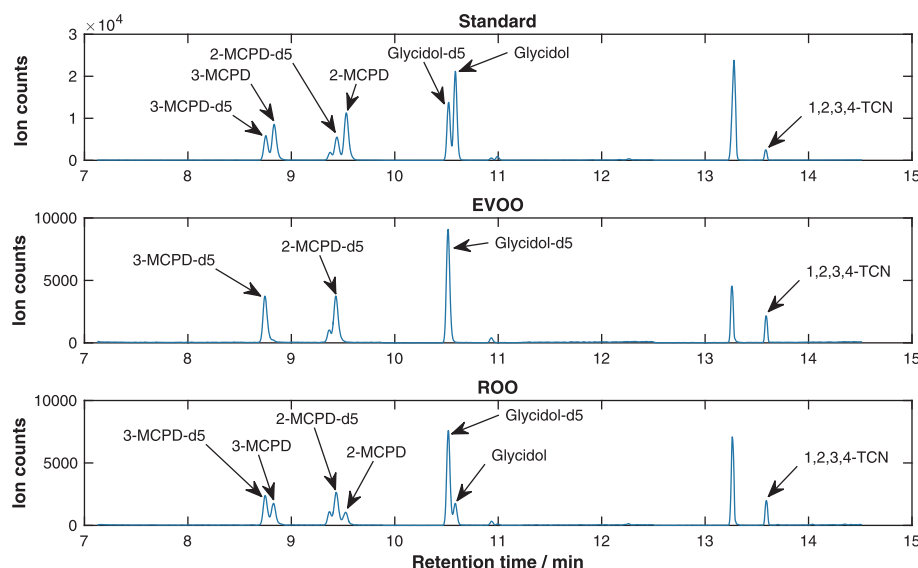


Fig. 1. GC-MS/MS chromatograms (TIC) of the PBA derivatives of the target compounds in a standard solution, an EVOO sample and a ROO sample.

Finally, the free contaminants are derivatized with phenylboronic acid prior to GC-MS/MS analysis.

The extraction method of the 2-, 3-MCPD esters and GEs, which was used in this experiment, followed basically the AOCS official method Cd29a_13 (AOCS, 2013) with some slight modifications. Firstly, GC-MS/MS was used for the measurement instead of GC-MS, for additional selectivity. Secondly, an extra internal standard PP-2-MCPD-d5 were applied, so that 2-MCPD could be quantified on its own internal standard, which improves the accuracy of 2-MCPD results. The third modification concerns the reconstitution of the dry residue in the final step in iso-octane instead of n-heptane. Fourthly, before transferring the supernatant to the GC vial, 50 μ L 1,2,3,4-TCN solution was added to each sample as a syringe standard. This syringe standard was used to monitor the GC injection reproducibility and sensitivity drift of the MS/MS instrument over time.

2.4. GC-MS/MS

The instrumental measurement was performed according to the AOCS official method Cd29a_13 (AOCS, 2013), but with a modification of using GC-MS/MS instead of GC-MS. GC-MS/MS analyses were carried out using a Varian CP-3800 GC and a Varian CP-8400 auto-sampler, combined with Varian 1200L Quadrupole MS/MS system from Varian (USA), equipped with an DB-35 ms fused-silica column (30 m \times 0.25 mm ID \times 0.25 μ m film thickness), purchased from Agilent (USA).

Gas chromatographic settings: The injector was set at 250 $^{\circ}$ C on pulsed splitless mode with a pulse pressure of 30.0 psi and a pulse duration of 1.20 min (Varian type 1079 EFC). 2 μ L samples were injected and carried by 1 mL/min He. The transfer line temperature was set at 300 $^{\circ}$ C. The GC oven temperature program started at 100 $^{\circ}$ C for 1 min, ramped to 160 $^{\circ}$ C at 20 $^{\circ}$ C/min, hold for 1 min at 160 $^{\circ}$ C, ramped to 180 $^{\circ}$ C at 4 $^{\circ}$ C/min, from 180 $^{\circ}$ C to 340 $^{\circ}$ C at 30 $^{\circ}$ C/min, hold for 4.70 min at 340 $^{\circ}$ C. The total run duration is 20.03 min.

Mass spectrometer settings: The ionisation was set at 70 eV. The monitored mass transitions are described in Table 2S.

All oil samples were analysed in duplicate, and data used were the average value of the two replicates of each oil sample.

2.5. Collision energy optimization

The most abundant daughter ions were identified by infusion of standard solutions of the PBA derivative of the target analytes. The transitions are mentioned in Table 2S. Afterwards, the optimal collision

energies were determined, and these values are mentioned in Table 2S. These mass transitions correspond to the PBA derivatives of the unbound native target compounds and their internal standards.

2.5.1. Quality assurance and quality control

The chemical analysis of the samples is performed according to the AOCS official method Cd29a_13 (AOCS, 2013). This method is implemented in our laboratory and experiments demonstrated that the method (with slight modifications as mentioned earlier) meets the method performance requirements as specified in Cd29a_13. Quality is controlled by analysis of blank samples, the use of deuterated internal standards, duplicate analysis of every individual sample and spiking experiments.

2.6. Statistical analysis

One-way analysis of variance (ANOVA) was performed to assess the differences in concentrations of the compounds between types of oils. Subsequently, Tamhane's T2 post hoc test was applied for pairwise comparisons. $P = .05$ was used throughout the study. The statistical analyses resulting in the figures were performed using MATLAB (R2015b, The MathWorks Inc., Natick, MA, USA). The ANOVA and post hoc test were carried out in XLstat (Addinsoft, New York, NY, USA).

3. Results and discussion

3.1. The QA of the measurements

Seven compounds (3-MCPD-d5, 3-MCPD, 2-MCPD-d5, 2-MCPD, Glycidol-d5, Glycidol, 1,2,3,4-TCN) were, after sample preparation, analysed by GC-MS/MS. The Total Ion Current (TIC) chromatograms of a standard solution, an EVOO sample, and a ROO sample, which were selected randomly from the large sample set, are presented in Fig. 1. 1,2,3,4-TCN is added to every sample prior to injection as an internal standard to monitor the injector and detector performance throughout the sample sequence.

The internal standards (e.g. 3-MCPD-d5), eluted prior to the native compounds (e.g. 3-MCPD). This is in agreement with previous reports (Abd Razak et al., 2012). From Fig. 1, it is clear that traces of 2-MCPD, 3-MCPD and glycidol could be detected in ROO, whereas they were below the limit of quantification in EVOO.

To illustrate assay accuracy, the standard curves were established using the optimized parameters in Table 2S. The results in Fig. 2 show the relationship between the ratio of the amount of 3-MCPD to the

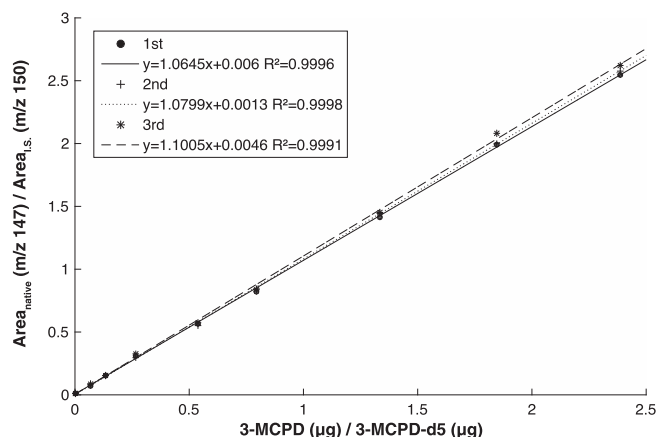


Fig. 2. Three calibration curves of 3-MCPD. X-axis is the ratio of the amount of standard (3-MCPD) to the amount of internal standard (3-MCPD-d5). Y-axis is the ratio of corresponding peak areas.

amount of 3-MCPD-d5 and the ratio of corresponding peak areas. In accordance with the AOCS method Cd 29a-13, 100 ± 10 mg of blank oil (containing no 2-MCPD/3-MCPD esters and GEs) were added to the calibration samples to improve the robustness of the method, since the oil matrix helps to retain the analytes during the evaporation step. The calibration curves of 3-MCPD were determined in triplicate between and after the whole sample series. The values of *a* and *b* of those three curves are quite similar only with small differences and all the values of *R*² are more than 0.999. According to the AOCS method criteria, the values of *b* and *R*² of the three calibration curves meet the requirements, which are $|b| < 0.02$, $R^2 > 0.99$. It demonstrates the stability of the measurement system, as well as a good repeatability and linearity of this method. The calibration curves were used to calculate the concentrations of the target compounds in the oil samples in the remainder of the study.

3.2. MCPD esters and GEs contents in relation to vegetable oil type

The 94 samples, including 30 EVOO, 18 ROO, 16 POO, 8C-VEGE, 12 R-VEGE, and 10 blends, were subjected to GC-MS/MS analysis. The results of the measurements of the 3-MCPD esters, 2-MCPD esters, and GEs in the five pure types of oils are presented in box plots (Fig. 3).

As shown in Fig. 3, the concentrations of the 2- and 3-MCPD esters and the GEs vary in the ranges 0–6 mg/kg, 0–1.5 mg/kg, and 0–1 mg/kg, respectively. The concentrations of 3-MCPD esters in samples are at least twice the concentrations of 2-MCPD esters. This may be due to the two positions (1- and 3-) of glycerol available to be chlorinated and resulting in 3-MCPD esters, compared with only one position (2-) resulting in 2-MCPD ester formation. There seems hardly any correlation between MCPDs and GEs concentrations in the oil samples. It is generally believed that GEs formation to be independent from MCPD ester formation (EFSA, 2016).

The mean concentrations of the three compounds and significant differences between oil types are presented in Table 1. Clearly, the concentrations of the three compounds in the cold-pressed oils (EVOO, C-VEGE) are significantly lower than in POO and ROO. R-VEGE shows values between these two groups. The differences between the olive oil grades and the vegetable oil grades are discussed in the following paragraphs.

3.2.1. Comparison of cold pressed olive oil and lower grades of olive oils

The concentrations of the three compounds in all individual oil samples are presented in Fig. 4. The data have been sorted according to the measured concentration of 3-MCPD esters. The 3-MCPD esters concentrations measured in the ROO and POO samples are considerable and significantly higher than in EVOO (Table 1). Experiments with

EVOO carried out by Matthäus and Pudiel (2013) revealed that in EVOO approx. 1 mg/kg 3-MCPD esters could be generated after 2 h of high temperature heating. On the other hand, Ozdikicierler, Yemiscioglu, and Gumuskessen (2016) detected no 3-MCPD esters and GEs in POO during steam distillation. These discrepancies with our current results are likely to be due to the fact that in our study samples of commercial origin were used and industrial processes had been applied to those samples. Moreover, it may be due to the degradation of the esters after a long time of heating (Ermacora & Hrncirik, 2014).

According to previous studies, several factors significantly promote the formation of 3-MCPD esters, such as temperature, heating time, pH value, moisture content, pressure and oil types (Hamlet et al., 2015; Ozdikicierler et al., 2016). High temperature is the main factor that can cause 3-MCPD esters and GEs formation (Abd Razak et al., 2012; Hrncirik & van Duijn, 2011). Previous studies also indicated that high temperature is employed in both ROO (Li et al., 2016) and POO (Moral & Mendez, 2006). Addition of a large amount of water in the degumming process and the use of high temperatures in the deodorization process could also attribute to the higher formation of glycidol in refined oil (Wang, Ji, & Han, 2017). Furthermore, the results of the current study reveal that the contents of 3-MCPD esters and GEs in POO are higher than in most of the ROO samples. Because the refining process takes place in both ROO and POO, the differences originate most probably from the phase before refining. There are three possibly reasons for the high concentrations, 1) an additional high temperature drying treatment happened before the refining process (Moral & Mendez, 2006; Ozdikicierler et al., 2016), 2) the extra exposed time of olive pomace to water, which results in the increased chance of monoacylglycerol (MAG) and diacylglycerol (DAG) formation which are precursors of bound 3-MCPD and glycidyl esters (Shahidi, 2005), 3) presence of more precursors in the oil (because of its lower quality) prior to refining.

3.2.2. Comparison of cold pressed and refined vegetable oils

In order to be able to compare the results of the olive oils, a small set of other cold pressed and refined vegetable oils were analysed as well (C-VEGE and R-VEGE). C-VEGE consisted of seven rapeseed oils and one sunflower oil, the latter of which is number four in Fig. 4. R-VEGE comprised three types of oils: the first three samples are rapeseed oils, number 4–6 and 12 are the peanut oils, number 7–11 are the sunflower oils.

It can be seen from Fig. 4 that the concentrations of C-VEGE are considerably lower than those of the R-VEGE samples, which is in agreement with the olive oil samples. The most likely explanation is, in line with the olive oils, the high temperature treatment during refining process (Abd Razak et al., 2012; Hrncirik & van Duijn, 2011). It also appears that the concentrations of those three compounds in the refined peanut oil samples have the tendency to be higher than in the other two types of seed oils (particularly the MCPD esters in sample 12 and GEs in sample 6), although the number of peanut samples was limited. The results may be due to the peanut ripeness level result in high DAG levels in peanut oils which are the precursors of the esters (Akhtar, Khalid, Ahmed, Shahzad, & Suleria, 2014; Ayres, 1983).

3.2.3. Comparison of olive oils and other vegetable oils

Both EVOO and C-VEGE are cold pressed oils, which means that during the whole processing, the extraction temperatures are controlled not exceeding 27 °C for EVOO (Boselli, Di Lecce, Strabbioli, Pieralisi, & Frega, 2009), 75–80 °C for rapeseed oil (Cvengros, 1995) and 38–40 °C for sunflower oil (Bendini et al., 2011). As shown in Fig. 4, there is no large difference between EVOO and C-VEGE: the value ranges of 3-MCPD esters, 2-MCPD esters and GEs are 0–0.08 mg/kg, 0–0.08 mg/kg and 0–0.15 mg/kg, respectively.

In general, the values of the 3-MCPD esters in ROO and POO are larger than in the other refined vegetable oils. Similar results were found in previous studies, which has already mentioned above,

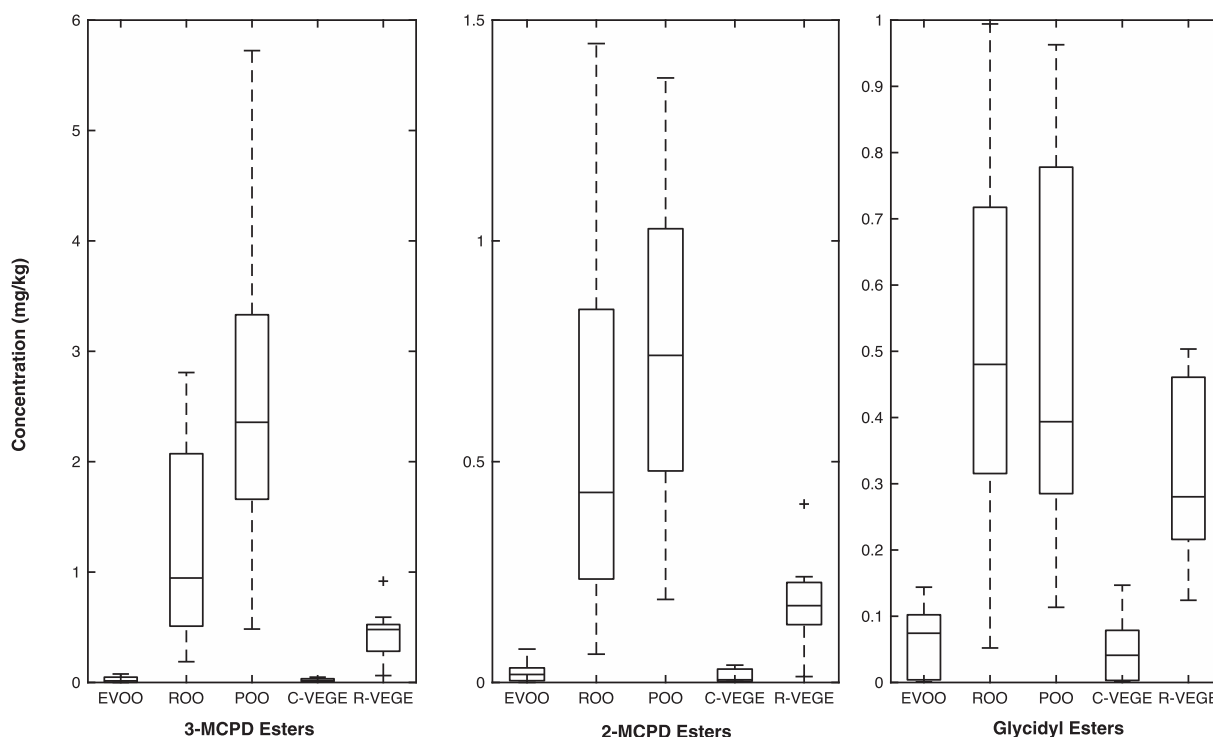


Fig. 3. Box plots of the measurements of the 3-MCPD esters, 2-MCPD esters, and GEs in the five types of oils. The central mark represents the median value, the edges of the line are the minimum and maximum values, and the edges of the box are first quartile and third quartile values for the five types of oil.

Table 1

Mean concentrations of the 3-MCPD esters, 2-MCPD esters, and GEs in the five types of oils and statistical comparisons.^a

| | 3-MCPD esters (mg/kg) | 2-MCPD esters (mg/kg) | GEs (mg/kg) |
|---------|-----------------------|-----------------------|---------------------|
| POO | 2.896 ^a | 1.031 ^a | 0.694 ^a |
| ROO | 1.210 ^a | 0.558 ^a | 0.516 ^a |
| R-VEGE | 0.427 ^b | 0.176 ^b | 0.426 ^{ab} |
| EVOO | 0.026 ^c | 0.021 ^c | 0.059 ^b |
| C-VEGE | 0.022 ^c | 0.015 ^c | 0.049 ^b |
| P value | < .0001 | < .0001 | .012 |

^a ANOVA and Tamhane's T2 post hoc tests; different superscripts in a column indicate significant differences.

indicating that olive oil and peanut oil generate higher levels of 3-MCPD esters than seed oils (rapeseed oil, sunflower oils) due to elevated DAG levels (Franke, Strijowski, Fleck, & Pudel, 2009; Hamlet et al., 2015; Ramli, Siew, Ibrahim, Kuntom, & Abd Razak, 2015; Zelinkova et al., 2006).

3.3. Authentication considerations

In order to evaluate the values of the three compounds for authentication practices, all data are presented together in Fig. 5a. This is a 3D scatter plot based on the measurement data of the 3-MCPD esters, 2-MCPD esters, and GEs. In the plot, one outlier sample showing the highest concentrations for all three compounds is lacking for legibility reasons. The EVOO samples, which are located in the origin point, are completely separated from ROO and POO in the three dimensions, see Fig. 5c and d. The plot also shows that 2-MCPD and 3-MCPD esters concentrations are fairly correlated for ROO but not so much for POO, which shows a more scattered pattern.

In order to examine potential matrix effects when examining mixtures, mixtures of EVOO and ROO or POO samples were analysed. These samples, with increasing lower grade oil concentrations show a gradual, linear change in MCPD esters and GEs concentrations (Fig. 5b:

mix1 and mix2): $R^2 = 0.98$ for ROO and $R^2 = 0.97$ for POO. Obviously, when mixing EVOO with lower grade olive oils, the concentrations of MCPD esters and GEs is fully determined by the fractions of the two types of oil.

In view of fraud detection, the 95% upper bounds of the EVOO sample set were subsequently calculated in order to set upper limits for real EVOO. This resulted in the following values: 0.036 mg 3-MPCD/kg oil, 0.028 mg 2-MCPD/kg oil, and 0.078 mg GE/kg oil. It is obvious that all 100% ROO or POO would be easily discriminated from the real EVOO, they are all exceeding these values considerably. However, the smarter fraudster will mix oils. The most difficult scenario would be the admixture of an EVOO with relatively low levels of the three compounds for the EVOO population with an ROO or POO with also low levels of the three compounds for their populations. Therefore, for this worst case scenario mixtures of EVOO at the 95% lower bound level and ROO or POO also at their 95% lower bound level were considered. The concentrations in EVOO at 95% lower bound are 0.016 mg 3-MPCD/kg oil, 0.014 mg 2-MCPD/kg oil, and 0.041 mg GE/kg oil; for ROO 0.804 mg 3-MPCD/kg oil, 0.365 mg 2-MCPD/kg oil, and 0.369 mg GE/kg oil; and for POO 1.804 mg 3-MPCD/kg oil, 0.479 mg 2-MCPD/kg oil, and 0.328 mg GE/kg oil. For calculation of the 'virtual' concentrations in the worst case scenario mixtures, a linear relationship was considered based on the results of the mixture analysis above. An example for the calculation of the 3-MCPD concentration in a mixture of EVOO and POO is shown below.

$$[3\text{-MCPD}(\text{mix})] = \frac{\text{Mass}(\text{EVOO})}{\text{Mass}(\text{EVOO} + \text{POO})} \times [3\text{-MCPD}(\text{EVOO})] + \frac{\text{Mass}(\text{POO})}{\text{Mass}(\text{EVOO} + \text{POO})} \times [3\text{-MCPD}(\text{POO})]$$

The resulting values for the mixtures were compared to the set upper limits, i.e. 0.036 mg 3-MPCD/kg oil, 0.028 mg 2-MCPD/kg oil, and 0.078 mg GE/kg oil (see above) in order to determine the lowest levels at which ROO or POO mixed into EVOO could be detected. ROO and POO admixtures to EVOO would be detectable at 2% w/w levels based on 3-MCPD esters, at 5% based on 2-MCPD esters, and at 13%

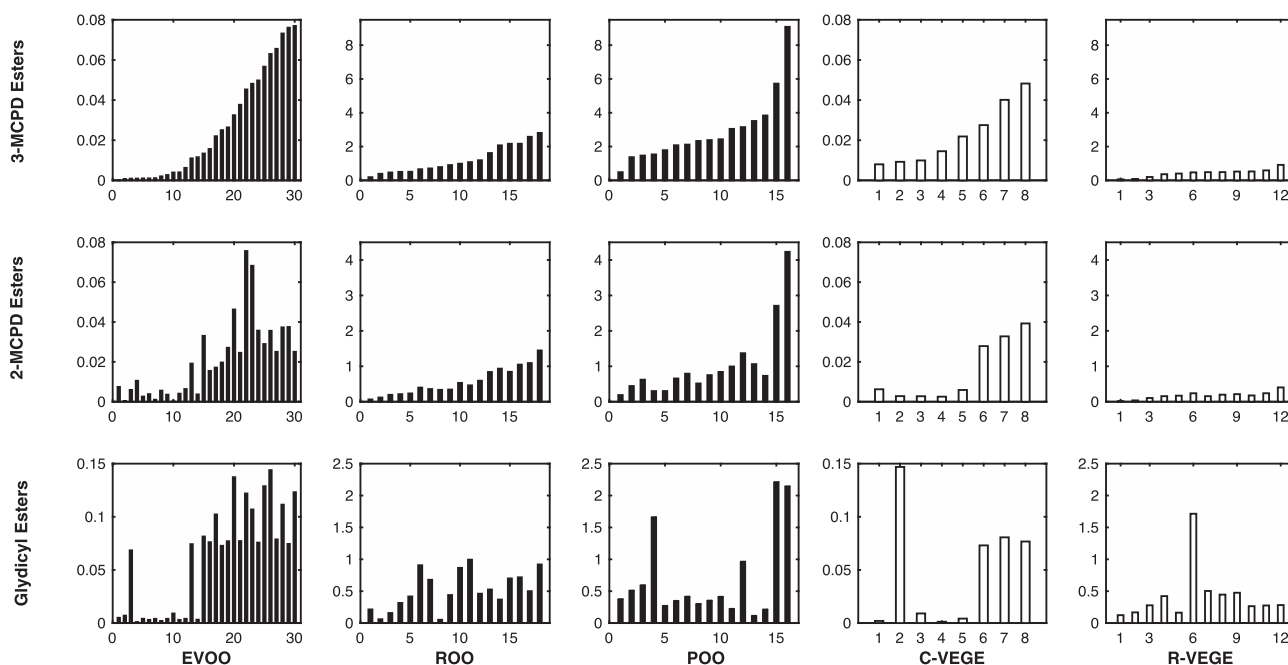


Fig. 4. The concentrations of 3-MCPD esters, 2-MCPD esters, and GEs in the individual samples of the five types of oils (mg/kg): Samples are sorted according to an increasing 3-MCPD esters content.

(ROO) and 14% (POO) based on GEs.

These results are very promising. MCPD esters seem a very sensitive marker group for detection of ROO or POO in EVOO. The methodology is robust, an internationally accepted method is applied, and the marker itself is robust from a fraud perspective too. It is hard to remove these compounds from ROO or POO. Certainly, a wider set of olive oils, including soft deodorized oils, as well as other vegetable oils will be

required for further confirmation. Furthermore, it has to be kept in mind that the manual sample preparation method is fairly labor intensive, although nowadays fully automated sample preparation robots, coupled to GC-MS(/MS) are available for quick and routinely analysis of oil samples allowing a wide application of such oil authenticity testing approach (Jacq et al., 2008; Parkinson, Bruheim, Christ, & Pawliszyn, 2004).

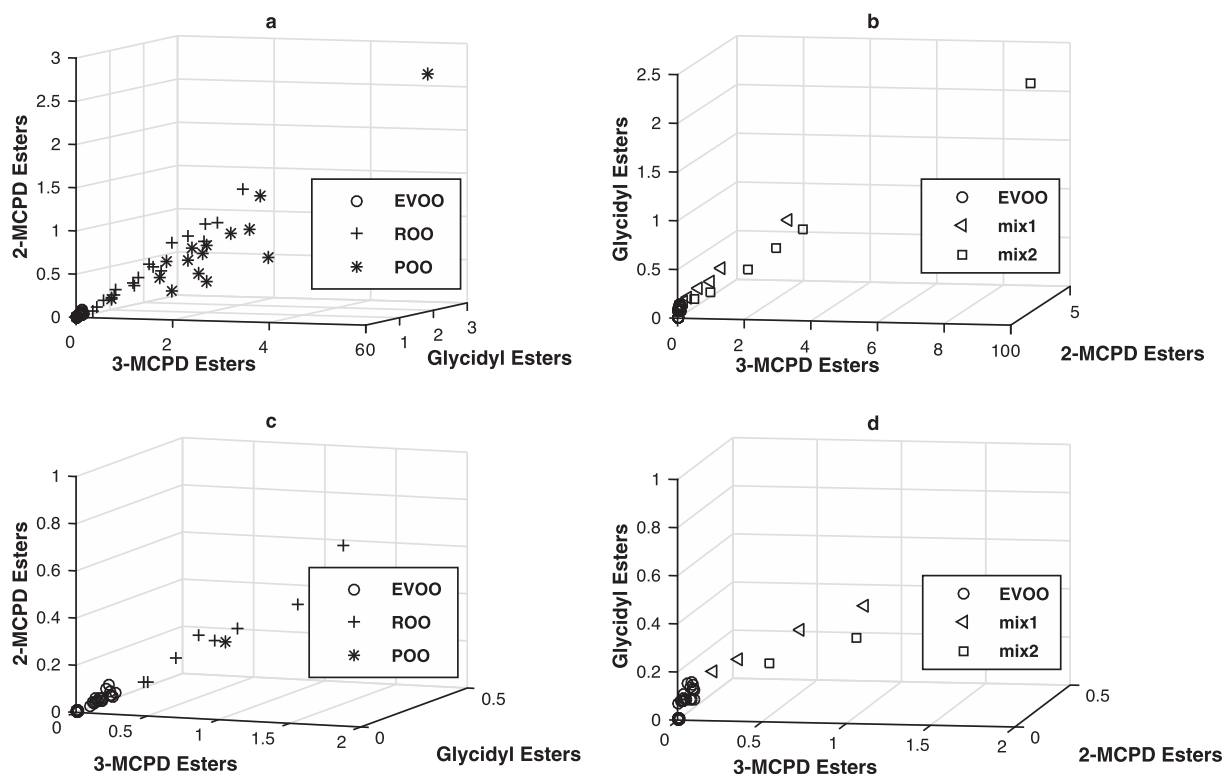


Fig. 5. Three dimension scatter plots of the three compounds in (a) EVOO, ROO, and POO; (b) the EVOO samples and mixtures of EVOO/ROO (mix1) and EVOO/POO (mix2); (c) zoomed in section of plot a; (d) zoomed in section of plot b (mix 1 refers to samples comprised 10%, 20%, 30%, 40% and 50% of ROO in EVOO. mix 2 refers to samples comprised 10%, 20%, 30%, 40% and 50% of POO in EVOO).

3.4. Safety considerations

As already mentioned MCPD esters have toxic effects, but what about the concentrations measured in the oils? Considering the TDI for 3-MCPD esters (0.8 µg/kg bw per day (EFSA, 2016), the intake for an adult person (60 kg) would amount 48 µg 3-MCPD esters per day. For the mean olive oil concentrations (Table 1), this intake would be theoretically reached by consumption of olive oil only with an intake of 1845.6 g EVOO/day, 39.6 g ROO/day (ca. 3 table spoons) or 16.8 g POO/day (ca. 1 table spoon). One can imagine that these ROO or POO volumes can be met in practice. For a full evaluation larger sets and more kinds of oils would need to be examined and compared to consumption data to understand the potential relevance of the 3-MCPD ester contamination in this kind of oils. In addition, 3-MCPD esters may be ingested through other foods, adding up to the total exposure. Nevertheless, it is obvious that the refined oils in the current study may contribute to the daily intake of 3-MCPD esters for users of these oils, and most likely to the intake of 2-MCPD esters and GEs as well.

4. Conclusions

The present study was designed to evaluate the value of MCPD esters and GEs for authentication of the premium processing grades of olive oils and other vegetable oils. Cold-pressed oils showed significantly lower levels of MCPD esters and GEs than their refined counterparts. Calculations revealed that 3-MCPD esters, 2-MCPD esters, and GEs would allow detection of adulteration of EVOO with 2%, 5%, and 13–14% ROO or POO (95% confidence) based on the current set. Therefore, this approach appears very promising and sensitive to detection of EVOO fraud with lower processing grade oils.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2017.12.025>.

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